correlation techniques drastically reduces the high detection limit in a modest time.

See also: **I/Chromatography. II/Chromatography: Liquid:** Theory of Liquid Chromatography. **Electrophoresis:** Capillary Electrophoresis.

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Countercurrent Chromatography and High Speed Countercurrent Chromatography: Instrumentation

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Introduction

This article presents a brief overview of the most significant aspects of the history, apparatus, theory and practice of countercurrent chromatography (CCC). CCC is primarily a preparative technique for the isolation and purification of chemicals on a milligram to multigram scale. It has been broadly applied to natural products, pharmaceuticals and other synthetic organic and inorganic chemicals.

What is Countercurrent Chromatography?

Countercurrent chromatography can be broadly characterized as a form of liquid–liquid chromatography (LLC) in which two mutually saturated immiscible liquids are employed. One phase is retained in the chromatograph as a long continuous or segmented stationary bed without the use of an absorptive matrix; the second phase passes through the stationary bed and is efficiently equilibrated with it by means of either hydrodynamic or turbulent mixing.

In earlier forms of liquid–liquid partition chromatography introduced by Martin and Synge, where one phase is retained in a porous matrix such as diatomaceous earth or cellulose, significant peak tailing is often seen and some analytes are lost by irreversible adsorption on the supporting matrix. In CCC, the stationary phase is retained by gravitational, inertial or capillary forces and adsorption is precluded by construction of the apparatus from polytetrafluoroethylene (PTFE) or other inert, usually polymeric, material. Thus analyte migration in CCC is determined only by its partition coefficient in the two-phase system and peaks are typically quite symmetrical.

CCC differs from countercurrent distribution (CCD) of the type introduced by Craig in the 1940s in that CCD is a discontinuous process based on attainment of partition equilibrium prior to phase transfer, whereas CCC is a continuous dynamic or steady-state process which characterizes all forms of chromatography. Both CCC and CCD may employ the same solvent systems and both achieve separations based on the partition coefficient, but the apparatus

employed and the mathematical treatment of each process are quite different.

The term CCC was coined by analogy to the earlier CCD process and is considered a misnomer by some because, as usually practised, one phase is stationary in the apparatus. However, the terms are justified by the consideration that if the observer resided in either one of the phases, without reference to the stationary apparatus, it would appear that the phases move in countercurrent fashion. Indeed it is possible to actually move both phases in opposite directions but it is then necessary to refer to the process by a redundant term such as 'true' CCC or 'dual' CCC.

Apparatus

Devices that today might be called countercurrent chromatographs were described as early as the 1930s and were used to separate lipophilic substances such as oil-soluble vitamins. However, the modern era of CCC began in the mid-1960s when Yoichiro Ito observed that if two immiscible liquids were placed in opposite halves of a closed helical coil and the coil was rotated on its axis, the liquids flowed into one another in countercurrent fashion (**Figure 1**). When placed at the initial interface, the individual components of a soluble sample migrate at different rates determined by their relative partition coefficients, K, in the two-phase system. If K, for example, is defined as concentration in the shaded phase divided by concentration in the unshaded phase shown in Figure 1 then components with K higher than unity will migrate to the right, while those with K less than unity will migrate to the left, and those with K equal to one will concentrate in a band at the initial interface. Ito's first CCC apparatus produced true countercurrent flow and was demonstrated to separate soluble mixtures of dyes and proteins in organic–aqueous systems, and also erythrocytes as an example of particulates that partition in two-phase aqueous polymer systems.

When the ends of the coil are opened, the system resembles an Archimedes' screw pump and both phases migrate in the same direction towards what is called the head end of the helix. The actual direction of flow depends on the handedness, left or right, of the coil and its direction of rotation, and will be towards the right side of the example shown in Figure 1. Now, if either one of the phases is pumped into the right end of the rotating coil in Figure 1, creating head-to-tail flow of the mobile phase, the other phase, attempting to flow towards the head, will be retarded by viscous forces and a very stable equilibrium will be established in which 40 to 60% of the unpumped phase will remain in the column as a stationary phase. Components of a sample introduced as a bolus in the mobile phase will partition between the mobile phase and the stationary phase and be eluted from the coil at the tail end in order of their partition coefficients. This process is exactly analogous to conventional column partition chromatography. As in other forms of chromatography, it is desirable to define the partition coefficient as

$$K = C_s/C_m$$

where C represents concentration in the stationary (C_s) and mobile (C_m) phases, respectively. Then substances with higher partition coefficients will be retained longer in the column.

Instrument geometries

Ito and colleagues have devised many ways in which a helical or modified helical coil of tubing can be rotated with respect to gravitational and inertial fields to achieve good retention of a stationary phase while minimizing band spreading and promoting efficient mass transfer of solutes. A few will be described here based on their historical or practical significance.

Horizontal flow-through coil planet centrifuge The behaviour just described in 'Apparatus' above is that produced by the horizontal flow through coil planet centrifuge (HFTCPC), first described in the late 1970s. Its characteristic motion is shown in **Figure 2B**. The device typically consisted of several single-layer helixes formed by winding PTFE tubing, measuring a few millimetres internal diameter, on rods about 12 mm in diameter and about 40 cm long, and mounting several of these, connected in series, on a cylindrical column holder. The holder was geared to rotate on its own axis, twice in each orbit, as it revolved around a central or solar shaft. Separations

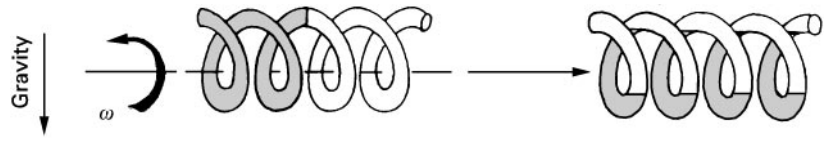

Figure 1 Countercurrent migration of immiscible liquids in a rotating helical tube with closed ends.

typically required 10 or more hours. The HFTCPC was not a commercial success at the time, but the configuration has reappeared as an optional column configuration in some recently described high-speed CCC apparatus.

Droplet countercurrent chromatography Earlier in the 1970s, Ito and colleagues described a simple non-centrifugal CCC technique called droplet countercurrent chromatography (DCCC). As illustrated in Figure 2A with a mobile heavy phase, it consisted of some 300 glass tubes, 1.8 mm i.d., connected in series with narrow-bore PTFE tubing. Either phase could be mobile. The technique, however, was limited to solvent systems that formed droplets. It relied on gravity for phase separation and was very slow, requiring 60 or more hours for a typical separation. In spite of these limitations, it was successfully marketed and was widely employed by natural product chemists; the technique is still available today.

Multilayer coil planet centrifuge The multilayer coil planet centrifuge (MLCPC) described by Ito and colleagues in the early 1980s is one of the most versatile and widely used instruments. Versions of it are available worldwide from several companies. It is shown schematically in Figure 2C, where the helical coil is seen to consist of several layers of tubing wound concentrically on the column holder, which again rotates twice on its axis in each orbit. The forcefield acting on the coil is quite different here from that obtained in the eccentric configuration (Figure 2B) just described. The orbital radius, R, is typically about 10 cm and the orbital frequency is typically about 800 r.p.m. The coil consists of about 10 to 16 layers of PTFE tubing, 2–3 mm i.d., wound on a spool 5 or more cm wide, starting at a radius r of about 5 cm (half of R) to a maximum of 8 or 9 cm. The winding is not actually a spiral as shown in Figure 2C, but consists of the multiple back-and-forth helical layers typically obtained by winding flexible tubing on a spool. A spool 5 cm wide and 17 cm in diameter might contain about 130 m of tubing 1.68 cm with i.d., with a total volume of about 300 mL.

The forcefield obtained in the MLCPC produces a unique mixing pattern in which the phases in the outward portions of the coil are separated as concentric layers of moving phase and stationary phase, the heavier layer being outwardly directed. On the other hand, in a segment of the inner portion of the coil, comprising about one-third of the coil volume, the phases are quite vigorously mixed. This dynamic mixing pattern is independent of the mobile phase flow rate and since the entire coil rotates through the mixing zone some 13 times each second, very good solute mass transfer is obtained. Typical separations are obtained in from 2 to 6 hours in 300-mL coils and

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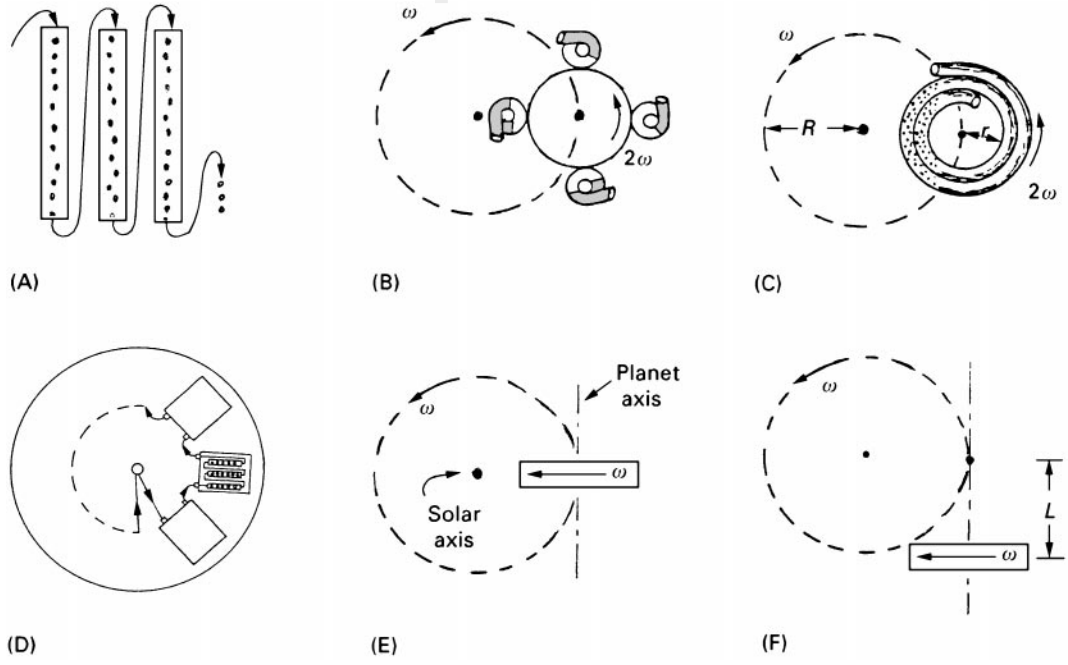

Figure 2 Schematic illustrations of (A) droplet countercurrent chromatography, (B) the horizontal flow-through coil planet centrifuge, (C) the multilayer coil planet centrifuge, (D) the centrifugal droplet countercurrent chromatograph or centrifugal partition chromatograph, (E) the cross-axis CCC and (F) the laterally displaced cross-axis CCC.

much faster separations are obtained, with some loss of resolution, in smaller coils. Resolution is echanced in the MLCPC by its very high retention of stationary phase, often 80% of the coil volume. A unique feature of all Ito-style CCC apparatus developed since about 1980 is the lack of a rotating seal. Because of the gear systems employed to rotate the columns, influent and effluent streams convey liquids from the instrument exterior to the rotating coil using a so-called antitwisting scheme, which does not involve a rotating seal, thereby avoiding leakage, local heating and contamination associated with such seals. Apparatus is available containing one, two or three spools on a single rotor and in some instruments multiple coils are available in a single spool. These may be operated independently or in series making the MLCPC one of the most versatile types of CCC apparatus.

Certrifugal partition chromatography One of the few types of modern CCC instrumentation developed outside Ito's laboratory was designed by Murayama and colleagues at Sanki Engineering (Japan) and was introduced in the early 1980s. It was designated simply as a centrifugal countercurrent chromatograph and today is usually referred to as a centrifugal partition chromatograph (CPC). Some confusion results from use of the CPC acronym since it is widely used to represent the coil planet centrifuge in Ito-style chromatographs. Conceptually the technique has been described as centrifugal droplet countercurrent chromatography (CDCCC, see Figure 2D). This description is based on the early models of the apparatus, which consisted of a series of cartridges of fluoroplastic in which a series of drilled cylindrical chambers were connected by small grooves, superficially resembling the larger gravitational DCCC apparatus. Cartridges were connected in series and spun at high speed in a rotor, to which inlet and outlet lines were connected through rotating seals. The apparatus could be filled with one phase and, when rotated, the other phase could be pumped through. Either the heavy (descending mode) or lighter (ascending mode) phase could be pumped through. In later versions of the apparatus, the chambers and passageways are formed by etching them in the surface of a sandwiched stack of rotor plates. Although described as a form of droplet chromatograph, actual droplet formation may not play a major role in mass transfer since many solvent systems function well in the CPC that do not form droplets in the gravitational apparatus. The apparatus is sometimes characterized as a static system, as opposed to a dynamic system, since mixing and mass transfer are dependent on the chamber design and on the mobile phase flow rate; mixing efficiency increases with increasing flow rate. An advantage of the CPC is that units of very large volume can be constructed since it is not subject to the problems of vibration associated with coil planet centrifuges.

Cross axis and laterally displaced cross axis CCC Since about 1989, Ito and colleagues have published many papers on chromatographs described as cross-axis, X-axis or laterally displaced cross-axis chromatographs (XL). It is difficult to illustrate these without three-dimensional models, but an attempt has been made to show them schematically in Figure 2E and F. In the X-axis unit, the coil axis is simply tilted 90°, or crossed with respect to the solar axis. In the earlier CCC units (Figure 2B and C), these axes are parallel. In the XL unit, the coil is further displaced a distance L along the planet axis from the central X position, where the solar axis lies in the rotational plane of the coil.

The effect of tilting the coil is to introduce a third dimensional component to the forcefield. In the parallel shaft units (Figure 2B and C), the forcefield vectors all lie in the two-dimensional plane of rotation of the coil. But the X and XL displacements introduce a force vector in the vertical or solar axis plane. These changes have a significant effect in improving phase mixing phenomena and column efficiency. This is particularly important for viscous solvent systems such as aqueous two-phase polymer systems of the polyethylene glycol-salt and polyethylene glycol-dextran type. These systems function poorly in the HFTCPC and even less well in the MLCPC systems. The aqueous two-phase polymer systems are important for the separation of proteins, enzymes and biological particulates. Unfortunately, the X and XL chromatographs are mechanically more complex than previous instruments and are not yet commercially available.

Monitors

Many CCC technicians simply collect fractions and monitor their composition by thin-layer chromatography (TLC). The column effluent can often be continuously monitored by UV absorption spectrophotometry. The main interference with monitoring is the tendency for droplets of stationary phase carried into the flow cell to cause noise spikes or to adhere to the cell windows. The problem is minimized by using a flow cell with a vertical flow path and by flowing a lighter mobile phase in the downward direction, and a heavier mobile phase in the upward direction. This tends to flush droplets of stationary phase through the cell quickly. Other methods used include warming the column effluent

just before it enters the monitor cell, or bleeding a stream of a miscible solvent, such as methanol, into the column effluent. Another approach uses a diode array detector to monitor the droplet noise in an area of the spectrum where the analyte does not absorb, such as the visible region, and to subtract the noise, on line, from the absorbance at the wavelength used for monitoring. Fluorescence may be used for monitoring and presents similar problems to absorptiometry.

Evaporative light-scattering detectors are excellent for monitoring compounds that lack chromophores and work with other compounds as well. The volatile solvents employed in CCC are quite compatible with evaporative light scattering as long as no non-volatile constituents like salts or buffers are incorporated into the solvent system. Several publications have appeared linking CCC with mass spectrometry and with nuclear magnetic resonance (NMR).

Theory

Countercurrent chromatography (CCC) is usually done by first filling the column at rest with stationary phase, then starting rotation and pumping in the mobile phase at a constant flow rate. The sample may be introduced at the head of the mobile phase stream or later. As it makes its initial pass through the coil, the mobile phase stream will displace a volume, V_{CO}, of stationary phase, which will be carried over into the collection vessel. Subsequently, the phase-volume ratio in the column is usually stable. This carryover volume is then a measure of the mobile-phase volume V_m and the stationary phase volume, V_s, can be estimated by subtracting V_{CO} from the known column volume V_C:

$$V_s = V_C - V_m = V_C - V_{CO}$$

Measuring the retention time of an unretained solute provides an alternative means for estimating V_m.

Because V_s is known in CCC, the chromatogram can be advantageously described in terms of the concentration partition coefficient K (defined earlier as $K = C_s/C_m$) of the analyte, rather than the quantity distribution coefficient or retention factor, k, used in other forms of chromatography.

The countercurrent chromatogram can be succinctly perceived visually in terms of K and the fact that an analyte with $K = 1$ will *always* elute with a retention volume equal to the column volume, V_C. The column volume, or the time corresponding to it, is the focal point of the countercurrent chromatogram and the emergence of $K = 1$ at this point is independent of the phase volume ratio. Of course, analytes with $K = 0$ will not be retained and will emerge with the mobile phase front. Other analytes with integral values of K will elute at multiples of the stationary phase volume, or time, beyond $K = 1$ and the position of intermediate values can be determined by linear interpolation. Therefore, **Figure 3** presents a completely general picture of a countercurrent chromatogram of analytes with $K = 0, 1, 2, 3$ and it is apparent that the elution pattern is described by the equation:

$$V_R = V_m + KV_s$$

where V_R is the analyte retention volume.

Since flow rate is usually constant, a time scale can be added or imagined. For instance if Figure 3 were obtained with a 300-mL column at a flow rate of 3 mL per min, $K = 1$ would emerge at 100 min and $K = 0$ at about 30 min. V_s then corresponds to 70 min and $K = 3$ emerges at about 240 min. An analyte with $K = 1.5$ could be expected to emerge at about 135 min. An example showing the separation of a series of phenylalkanols is shown in **Figure 4**.

Separation of the closely related compounds paclitaxel and cephalomanine by a form of recycling CCC is shown in **Figure 5**. In this approach, the peak containing the mixture was separated from other extraneous material in the first cycle through the CCC by shunting the effluent containing it into a holding tube of the same diameter tubing used in the CCC column. The shunt was then reconnected to the CCC inlet and the material chromatographed a second time, and the shunting process was again repeated to obtain the separation shown in Figure 5.

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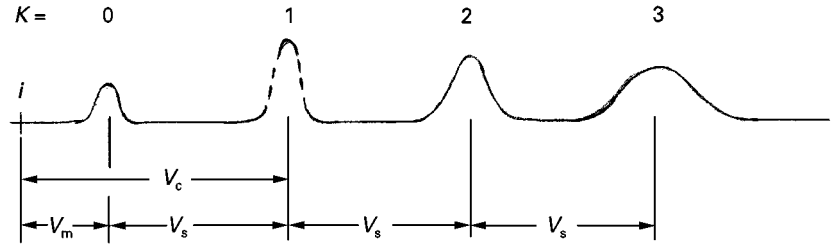

Figure 3 A generalized countercurrent chromatogram for solutes with partition coefficients, K, of 0, 1, 2 and 3.

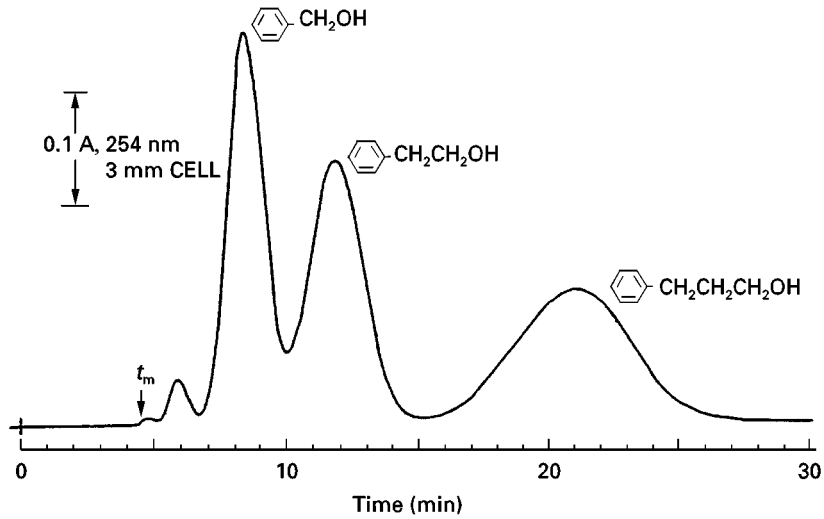

Figure 4 CCC separation of a mixture of 10 μL each of benzyl alcohol, 2-phenylethanol and 3-phenylpropanol on a 24-m coil of 1.68-mm PTFE, 56 ml volume using heptane as stationary phase and a mobile phase of 25% 2-propanol in water flowing at 4 mL min^{-1}. Reproduced from Conway WD, Buchert EL, Sarlo AM and Chan CW (1998) with permission from Marcel Dekker.

Resolution

The effect of V_s on analyte resolution is also apparent from Figure 3. Experimental parameters that alter V_s, such as flow rate or rotational speed, will not affect the $K = 1$ point. But as flow rate is increased, more stationary phase will be carried over and the V_m distance will increase towards the right, decreasing V_s. The V_s distances beyond $K = 1$ will move to the left, thereby decreasing resolution between the peaks. In the extreme case of high flow rate, all stationary phase will be forced from the column and all peaks will converge and be eluted at the column volume, or $K = 1$ point, which emphasizes the importance of the column volume or $K = 1$ point in the countercurrent chromatogram.

Direct measurement of the resolution, R_s, of two peaks on a chromatogram is based on the definition:

$$R_s = \frac{V_2 - V_1}{0.5(W_2 + W_1)}$$

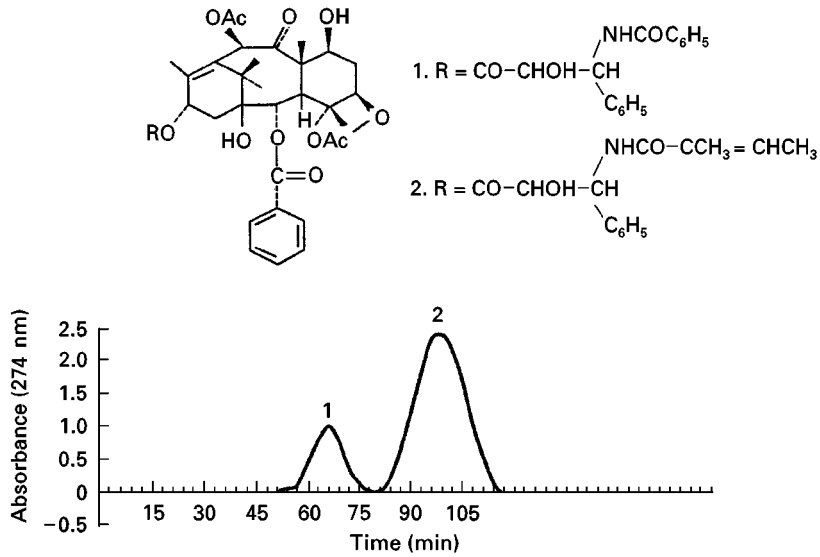

Figure 5 Separation of paclitaxel (1) and cephalomanine (2) by recycling CCC using the solvent system hexane/ethyl acetate/methanol/water – 6 : 4 : 5 : 5 (v/v/v/v) and a 230-mL column of 1.6 mm PTFE with a mobile aqueous phase flowing at 1.7 mL min^{-1}. Reproduced from Du Q-Z, Ke C-Q and Ito Y (1998) with permission from Marcel Dekker.

where V_2 and V_1 are the retention volumes of the second and first peaks to emerge and W_2 and W_1 are their 4-sigma base widths measured in the same units as V_2 and V_1. With this definition an R_s value of 1.5 represents baseline resolution.

The contribution of other factors to resolution in CCC is summarized in the following general resolution equation:

$$R_s = 0.25(\alpha - 1)N^{0.5}\left[\frac{K_1}{0.5K_1(\alpha + 1) + (1 - S_F)/S_F}\right]$$

where K_1 is the partition coefficient of the first of a pair of chromatographic peaks to emerge and α, N and S_F are the separation factor, the column or coil efficiency, and the fraction of the column volume occupied by stationary phase, respectively.

The separation factor is defined as:

$$\alpha = K_2/K_1$$

where K_2 is the partition coefficient of the second peak of the pair to emerge and is always calculated to be greater than 1.

Efficiency in theoretical plates is defined in terms of the peak base width as:

$$N = 16\left(\frac{V_R}{W_1}\right)^2$$

where W_1 is the 4-sigma base width of the peak and is measured in the same units as V_R.

S_F, the stationary phase fraction is:

$$S_F = V_s/V_C$$

and its expression in the denominator of the resolution equation is seen to represent the phase-volume ratio:

$$(1 - S_F)/S_F = V_m/V_s$$

The resolution equation confirms the visual observation from Figure 3 that increasing V_s will increase resolution.

The three terms in the resolution equation are usually called the separation factor term, the efficiency term and the large term in brackets is the partition coefficient term. Each can be evaluated independently from the countercurrent chromatogram.

The α term The separation factor term depends on the selectivity of the solvent system for one analyte versus another. It is often the dominant term leading to separation but it is not predictable and must be determined by an empirical examination of various solvent systems. The search is best conducted by non-CCC means of measuring partition coefficients, such as high performance liquid chromatography (HPLC) or TLC or by using small-volume CCC columns. An advantage of CCC is that a very large number of solvent systems is available.

The efficiency term The square root dependence of N indicates that doubling resolution will require a four-fold increase in column length, which is often not practical because of the resultant increase in backpressure and separation time. CCC provides only a modest number of theoretical plates, usually in the range of 300 to 1000, unless narrow-bore columns are employed at low flow rate. It is comparable to flash-column chromatography in terms of N. However, resolution in CCC is frequently much better than might therefore be expected because of the greater contribution of α and because of the very high S_F, typically 0.7 or more, obtained with some solvent systems.

The partition coefficient term Changing the polarity of the solvent system to increase K_1 will increase resolution, but the benefit rapidly diminishes as K_1 increases. The magnitude of the effect depends on S_F, but when S_F is 0.4, 80% of the effect of increasing K_1 will be obtained when K_1 reaches about 4, at which retention time will be quite long, especially for K_2.

If S_F is 0.8, 80% of the effect of increasing K will be obtained when K_1 is about 1, so it is more practical to arrange conditions so that S_F is as high as possible and then adjust K of the component of interest to the range of about 1 to 2.

Solvent Systems

A very wide range of two-phase solvent systems can be used in CCC. These include an aqueous phase with solvents ranging in polarity from hexane through chloroform, ethyl acetate and n-butanol. Partition coefficients can be modified by mixing the organic solvents with each other or by adding a miscible solvent such as methanol, 2-propanol or acetonitrile to the system. While these dissolve primarily in the aqueous phase, they partially dissolve in the organic phase, making the system more miscible and shifting the analyte partition coefficient towards 1. When systems become too miscible, the stationary phase is poorly retained. An example is 2-butanol, which when saturated contains about 40% water by weight. Some solvent systems that have been extensively employed in CCC are given in **Table 1**.

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Table 1 Representative solvent systems for CCC

1. Hexane/ethyl acetate/methanol/water – 3 : 7 : 5 : 5 (v/v/v/v)
2. Chloroform/methanol/water – 7 : 13 : 8 (v/v/v)
3. Chloroform/methanol/water – 4 : 4 : 3 (v/v/v)
4. Chloroform/methanol/water – 5 : 5 : 3 (v/v/v)
5. Hexane/methanol – any ratio
6. Hexane/acetonitrile – any ratio
7. n-Butanol/water – any ratio

The first system given in Table 1 is useful for lipophilic materials; the chloroform systems are widely used for solutes of intermediate polarity and n-butanol/water is one of the most polar systems available. Systems 5 and 6 (Table 1) are non-aqueous systems and are particularly useful for very lipophilic materials with no solubility in water. Prior to its use in the CCC, the solvent system is always shaken in a separation funnel and allowed to separate to ensure mutual saturation of the two phases. Solvent systems are covered extensively in all of the reference works listed in Further Reading and are only discussed here very briefly.

Screening solvent systems

To use CCC successfully it is necessary to empirically search for suitable solvent systems using non-CCC methods initially. The first goal is to find a system that provides a K of 1 to 2 and that provides good sample solubility *in both phases*. This latter point is important because sample capacity will be limited by the phase in which the sample is least soluble. Two approaches to screening K values will be described here briefly.

Using TLC Prepare a small amount of the proposed bi-phasic solvent system and partition some sample between about 200 μL of each phase. Spot 20 μL of each of the phases side by side on a TLC plate and develop with a suitable TLC system. Visual examination, or better, scanning with a TLC densitometer, of the separated spots for the two phases will allow a judgement to be made as to which of the components have K values in the neighbourhood of 1.

Using HPLC Dissolve a small amount of sample in about 500 μL of aqueous phase. Chromatograph 20 μL in a suitable HPLC system. Then equilibrate the aqueous solution with the same volume of non-aqueous phase, discard the non-aqueous extract and again chromatograph 20 μL of the aqueous phase. From the respective peak heights, the partition coefficients in the CCC solvent system can be calculated.

Since CCC retention depends only on K, using the equation for V_R, or simply referring to Figure 3, the expected retention times of the sample components can be calculated.

Other Chromatographic Approaches

Substances with high K values that are not eluted, will be to some extent separated along the column and can be recovered by stopping the apparatus and pumping out the column contents. An alternative approach is to switch phases without stopping the chromatography. In the MLCPC, the appropriate directions for pumping the phases is head to tail for the heavy phase and tail to head for the lighter phase, so this approach involves switching the column inlet as well. For the CDCCC, the corresponding directions are downward for heavy phase and upward for lighter phase.

Yet another approach to manipulating CCC is to fill the column initially with a non-equilibrium ratio of phase volumes by simultaneously filling both phases at once. Retention of particular K values can be enhanced or diminished by choosing the appropriate phase-volume ratio. Gradients can also be used for a number of systems, particularly where the composition of the stationary phase changes very little as the polarity of the mobile phase is changed.

For acidic and basic substances, adjustment of pH or use of a pH gradient can be useful. The range of pH is not a limitation as far as the apparatus is concerned. Ion-pair formation and use of liquid-ion exchangers can also be exploited, and various chelating agents have been used for the separation of metals, particularly rare earths. Optical resolutions have been achieved by addition of chiral selectors predominantly soluble in one of the phases to the solvent system.

The above examples indicate a few of the many ways in which CCC apparatus can be manipulated to achieve separations by means other than straightforward chromatography.

pH-Zone Refining

The technique of pH-zone-refining CCC primarily developed by Weisz and Scher in collaboration with Ito, deserves special mention because it is unique to CCC. It is applicable to organic acids and bases and is clearly the technique of choice for obtaining highly purified dyes and samples of the impurities contained therein.

For a very brief explanation it is convenient to view the technique as a kind of two-phase acid–base titration carried out in a CCC column. To illustrate this, imagine a series of aromatic carboxylic acids that

differ slightly in pK_a as well as lipophilicity. Typically a simple solvent system such as methyl t-butyl ether (MTBE) and water is mutually saturated and the phases put in separate containers. The organic phase is acidified with trifluoroacetic acid (TFA) and the column is filled with it as the stationary phase. In practice, either phase can serve as the stationary phase but the organic phase is chosen in this example. The sample of mixed acids is dissolved, or suspended if incompletely soluble, in a portion of the acidified stationary phase and introduced at the column inlet. The aqueous phase is made basic with ammonia and pumped into the rotating column. The acidified sample will dissolve in a relatively small zone of acidified MTBE at the column inlet and as the basic mobile aqueous phase is introduced it will neutralize portions of the sample, which will then be extracted into the aqueous phase. However, on moving down the column, the zone will encounter more TFA, undergo re-acidification and again be transferred to the stationary organic phase. This neutralization and transfer will proceed, as in a titration, first with the acid of lowest pK_a, modified also by the lipophilicity of the particular acid, those with lowest pK_a and lowest lipophilicity migrating fastest.

Monitoring the eluent by UV absorption is usually not informative, but monitoring the pH shows a series of broad steps of increasing pH containing very pure samples of the individual acids with impurities concentrated at the interfaces between the zones. Bases can be separated in a similar fashion. This technique provides a sample capacity of several grams.

An example showing the separation of two isomeric nitrochloromethoxybenzoic acids by pH-zone refining is presented in **Figure 6**. Monitoring the UV absorbance of the effluent yields only a flat trace, which does not indicate the separation. However, the effluent pH shows sharp steps at the start and end of the component zones. Using a 310 mL-column and a sample size of 20 g, the separation yielded 9 g of the pure 2-nitro compound, 5.3 g of the 6-nitro isomer and 2 g of a mixture of the two.

Conclusion

Modern CCC is a novel form of preparative LLC that offers more versatility in the choice of solvents, ranging from very lipophilic to moderately polar organic or aqueous systems. It provides predictable retention behaviour and complete recovery of sample since retention depends only on the solute partition coefficient and adsorption is precluded by the inert nature of the apparatus. Its separation capability is further enhanced by various technical manipulations of solvent composition and flow during chromatography, which cannot be reproduced in other types of chromatography. CCC has been widely employed by

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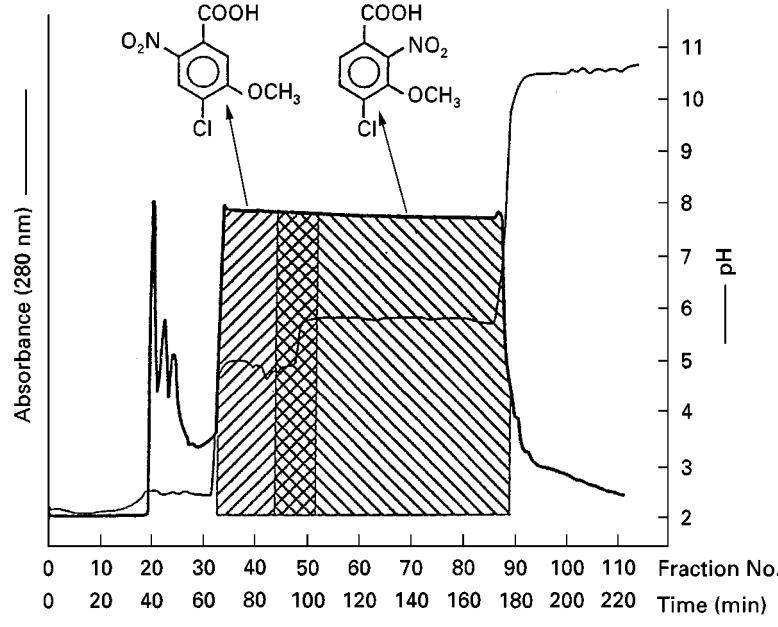

Figure 6 Separation of 2- and 6-nitro-4-chloro-3-methoxybenzoic acids by pH-zone-refining CCC. Initial stationary phase is 0.3% (12 mM) trifluoroacetic acid in the upper organic phase of a mixture of methyl t-butyl ether/acetonitrile/water – 4 : 1 : 5 (v/v/v). The mobile phase was 0.8% (100 mM) ammonia in the lower aqueous phase pumped at 3 mL min^{-1}. The column volume is 310 mL. Reproduced from Dudding T, Mekonnen B, Ito Y and Ziffer H (1998) with permission from Marcel Dekker.

natural product chemists for the isolation and purification of unstable bioactive materials from complex matrices. The technique deserves more attention from analytical chemists confronted with the separation of complex mixtures, synthetic chemists facing purification of non-crystalline products, diastereomers or optical resolution of racemates, and physical chemists seeking new approaches to the study of solvent interactions or the measurement of chemical properties such as partition coefficients or dissociation constants. Continuing study of new applications of CCC in several laboratories, as well as the commercial development of more reliable and user-friendly apparatus, promises to extend the use of CCC in the foreseeable future.

CCC Literature

References to CCC applications are found throughout the analytical and natural product literature and in the monographs cited below. A large number of references are found in *Journal of Chromatography* and in *Journal of Liquid Chromatography and Related Techniques*, many of which are collected in periodic special issues of these journals, the latest of which contains over 20 articles. A list of earlier special issues can be found in Conway (1995).

See also: **III/Alkaloids:** High-speed Countercurrent Chromatography. **Antibiotics:** High-speed Countercurrent Chromatography. **Chiral Separations:** Countercurrent Chromatography. **Dyes:** High-speed Countercurrent Chromatography. **Ion Analysis:** High-speed Countercurrent Chromatography. **Natural Products:** High-speed Countercurrent Chromatography. **Proteins:** High-speed Countercurrent Chromatography.

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Detectors: Laser Light Scattering

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Introduction

There are two types of light scattering detectors used in liquid chromatography, the evaporative light scattering detector and the liquid light scattering detector. The former evaporates the solvent from the column eluent in a gas stream and measures the light scattered by the residual solute particles; this type of detector is described elsewhere in this encyclopedia. The second, the liquid light scattering detector, senses the actual solute molecules themselves by light scattering measurements. Liquids containing large molecules such as synthetic polymers, biopolymers, e.g. polypeptides, proteins and polysaccharides, scatter light and, providing the incident light is strong enough, the scattered light can be sensed and used to detect the presence of the solute. In practice the column eluent is allowed to flow though a cell through which passes a high intensity beam of light. The light source is usually a parallel beam laser (light amplification by